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Guanosine pentaphosphate phosphohydrolase of *Escherichia coli* is a long-chain exopolyphosphatase

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ABSTRACT **An exopolyphosphatase [exopoly(*P*)ase; EC 3.6.1.11] activity has recently been purified to homogeneity from a mutant strain of *Escherichia coli* which lacks the principal exopoly(*P*)ase. The second exopoly(*P*)ase has now been identified as guanosine pentaphosphate phosphohydrolase (GPP; EC 3.6.1.40) by three lines of evidence: (*i*) the sequences of five tryptic digestion fragments of the purified protein are found in the translated *gppA* gene, (*ii*) the size of the protein (100 kDa) agrees with published values for GPP, and (*iii*) the ratio of exopoly(*P*)ase activity to GPP activity remains constant throughout a 300-fold purification in the last steps of the procedure. The enzyme liberates orthophosphate by processive hydrolysis of the phosphoanhydride bonds of polyphosphate [poly(*P*)] chains (1000 residues) or by hydrolysis of the 5′-γ-phosphate of guanosine 5′-triphosphate 3′-diphosphate (pppGpp) to guanosine 5′-diphosphate 3′-diphosphate (ppGpp or "magic spot"). The K_m for long-chain poly(*P*) as a substrate (≈0.5 nM) is far lower than that for pppGpp (0.13 mM); the k_{cat} for the poly(*P*)ase activity is 1.1 s^{-1} and that for pppGpp hydrolase is 0.023 s^{-1}. These and other findings direct attention to possible functions of poly(*P*) in the response of *E. coli* to stresses and deprivations.**

Inorganic polyphosphate [poly(*P*)] is a linear chain of nearly 1000 phosphoanhydride-bonded residues of inorganic phosphate (P_i) found in abundance in bacteria, fungi, plants, and animals (1, 2). Poly(*P*) has been implicated as a reservoir of energy (1, 3) and phosphate (3–5), as a kinase donor for sugars (1, 6, 7), in the chelation of cations (4, 8, 9), in the entry of DNA into bacterial cells (10), and in the regulation of gene expression and enzyme activity (1, 3, 11). Although some of these functions remain largely unproven, the ubiquity and dynamic features of poly(*P*) suggest a variety of important roles in cellular metabolism and organismal development.

Guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp) have long been implicated in regulation of bacterial adjustments to stress. In response to amino acid starvation in *Escherichia coli*, the cellular content of ppGpp increases, with a concomitant shift in transcription from ribosomal genes to biosynthetic genes, a phenomenon known as the stringent response (12, 13). RNA polymerase has been suggested as a target for regulation by ppGpp (12, 14).

Recently, poly(*P*) kinase (PPK), which reversibly transfers the terminal phosphate of ATP to form poly(*P*), was purified from *E. coli* (15). In the course of cloning and overexpressing the *ppk* gene, exopolyphosphatase (PPX; EC 3.6.1.11), which hydrolyzes the terminal residues of poly(*P*), was discovered in the same operon (16). From a *ppx* mutant a second poly(*P*)ase was purified and characterized. We report here its identification as guanosine pentaphosphate phosphohydrolase (GPP; EC 3.6.1.40), the protein which hydrolyzes the 5′-γ-phosphate of pppGpp to form ppGpp.

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MATERIALS AND METHODS

Reagents and Proteins. Sources were as follows: ATP, ADP, nonradiolabeled nucleotides, poly(*P*)s, bovine serum albumin, and ovalbumin from Sigma; [γ-^{32}P]ATP at 6000 Ci/mmol (1 Ci = 37 GBq) and [γ-^{32}P]GTP at 6000 Ci/mmol from ICN; Q-Sepharose fast flow, catalase, aldolase, Superose-12 fast protein liquid chromatography (FPLC) column, and Chromatofocusing column and reagents from Pharmacia LKB; DEAE-Fractogel, P11 phosphocellulose, and DE52 DEAE-cellulose from Whatman; protein standards for SDS/PAGE from Bio-Rad; and polyethyleneimine (PEI)-cellulose TLC plates from Merck. PPK (0.66 mg/ml, 30,000 units/μg) was prepared as described in ref. 18; PPX (0.09 mg/ml, 22,000 units/μg) was prepared as described in ref. 16. A poly(*P*) ladder (10–20 residues in length) was prepared by incubating [^{32}P]poly(*P*) (made *in vitro* by using PPK) in 10 mM HCl (pH 2) at 95°C for 5 min.

Bacterial Strains. *E. coli* CA10 (Δ[*lac–proAB*], *supE, thi*, F′[*traD36, proAB*$^+$, *lacI*^{Q}, *lacZ*ΔM15], *ppk*::*kan*) was constructed by M. Akiyama and E. Crooke.

Preparation of [^{32}P]Poly(*P*). [^{32}P]Poly(*P*) was prepared as described (15, 17). The reaction mixture contained 50 mM Hepes–KOH (pH 7.2), 40 mM $(NH_4)_2SO_4$, 4 mM magnesium acetate, 66.7 mM creatine phosphate, creatine kinase at 100 μg/ml, 1 mM ATP, 300 μCi of [γ-^{32}P]ATP (10 mCi/mmol), and 44 μl of PPK in a volume of 20 ml. The mixture was incubated at 37°C for 45 min. Progress of the reaction was determined by separating [γ-^{32}P]ATP from [^{32}P]poly(*P*) on PEI plates in 1 M LiCl and 1 M HCOOH, cutting the plates, and measuring the radioactivity on the strips by liquid scintillation counting. A 40% incorporation of [γ-^{32}P]ATP into poly(*P*) was obtained in approximately 1 h. The reaction was stopped by adding EDTA to 50 mM. The mixture was extracted once with phenol/chloroform (1:1, vol/vol) and three times with chloroform. Poly(*P*) was precipitated with 2 vol of 2-butanol at −20°C for 30 min and collected by centrifugation at 48,400 × *g* for 60 min. The pellet was dissolved in H_2O, and the poly(*P*) was precipitated twice more to remove unincorporated [γ-^{32}P]ATP, washed with ice-cold 67% (vol/vol) acetone, dried under reduced pressure, and then dissolved in H_2O to a final concentration of 1 mM in P_i residues.

Poly(*P*)ase Assay. The reaction mixture contained 25 mM Hepes-KOH (pH 8.0), 0.5 mM magnesium acetate, 0.5 mM dithiothreitol (DTT), 66.7 mM ammonium acetate, and 66.7 μM (in phosphate residues) [^{32}P]poly(*P*) in a final volume of 15 μl. The mixture was incubated for 30 min at 37°C. A 1-μl sample was spotted on a PEI plate, which was developed with

Abbreviations: DTT, dithiothreitol; GPP, guanosine 5′-triphosphate 3′-diphosphate phosphohydrolase; PEI, polyethyleneimine; poly(*P*), polyphosphate; poly(*P*)ase, polyphosphatase; PPK, poly(*P*) kinase; ppGpp, guanosine 5′-diphosphate 3′-diphosphate (guanosine tetraphosphate); pppGpp, guanosine 5′-triphosphate 3′-diphosphate (guanosine pentaphosphate); PPX, exopolyphosphatase [exopoly(*P*)ase].

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0.4 M LiCl and 1 M HCOOH. ADP and ATP (12.5 nmol each) were spotted at the origin as markers for the separation. The plates were cut according to the markers (origin, ATP, ADP), and the products were measured by liquid scintillation counting. One unit of activity liberates 1 pmol of $[^{32}P]P_i$ per min.

Preparation of [^{32}P]pppGpp. [5′-γ-^{32}P]pppGpp was prepared by using a ribosome-dependent enzymatic synthesis (18, 19). The 500-μl reaction mixture contained 100 mM Tris acetate (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 10 mM creatine phosphate, creatine kinase at 40 μg/ml, 4 mM ATP, 60 mM ammonium acetate, 2.2 mM [γ-^{32}P]GTP (50 cpm/pmol), and an *E. coli* ribosome preparation (compliments of Mitchel Singer, Stanford University). The mixture was incubated at 30°C for 1 h, extracted once with phenol/chloroform (1:1), adsorbed to a 1-ml DEAE-Fractogel column, and eluted with a gradient from 100 to 500 mM LiCl in 50 mM Tris·HCl (pH 7.5). Separation of nucleotides was monitored on 10-cm PEI plates (previously washed in methanol) developed in 1.5 M H_3PO_4/KH_2PO_4 (pH 3.5). [^{32}P]-pppGpp was precipitated from pooled fractions by adding LiCl to a concentration of 1 M and then 2 vol of 95% (vol/vol) ethanol. The precipitate was collected by centrifugation and dissolved in water.

pppGpp Hydrolase Assay. The reaction mixture (15 μl) containing 25 mM Hepes–KOH (pH 8.0), 0.5 mM magnesium acetate, 0.5 mM DTT, 66.7 mM ammonium acetate, and 220 μM [γ-^{32}P]pppGpp was incubated for 30 min at 37°C. A 2-μl sample of the reaction mixture was spotted on a PEI plate, which was developed with 0.4 M LiCl and 1 M HCOOH. ADP and ATP (12.5 nmol each) were spotted at the origin as markers for the separation. The plates were cut according to the markers (origin, ATP, ADP), and the products were measured by liquid scintillation counting. One unit of activity liberates 1 pmol of $[^{32}P]P_i$ per min.

Protein Sequencing. Fraction VI was precipitated with 10% trichloroacetic acid, washed with cold 100% acetone, and dried. The protein was resuspended in 10 μl of 8 M urea, mixed with a vortex mixer, and kept for 5 min at room temperature; 10 μl of 1 M Tris·HCl (pH 8.5) and 80 μl of H_2O were added to the mixture. Trypsin was added to the resuspended protein in the weight ratio of 1:20. The reaction was incubated at 37°C for 24 h with intermittent mixing and then stopped with 0.1% trifluoroacetic acid. The fragments were separated by hydrophobic HPLC. Five symmetrical peaks were sequenced with an Applied Biosystems 470 gas-phase protein sequencer with on-line HPLC by James Kenny and Alan Smith of the Protein and Nucleic Acid Facility at Stanford University.

RESULTS

Purification of the Second Exopoly(*P*)ase Activity. The procedure used *E. coli* CA10, a strain mutant in the *ppx* gene. After the heat lysis/sonication step, nucleic acids were removed by passing the supernatant over a DE52 column (Table 1). Media used for subsequent column chromatography included phosphocellulose, Fast Flow Q Sepharose, and Chromatofocusing. The activity was purified 3100-fold (Table 1). Assays of poly(*P*)ase and pppGpp hydrolase activities of fractions III to VI indicated an essentially constant ratio of activities over this 300-fold range of purification. The monomer molecular mass of GPP was estimated to be 50 kDa by SDS/PAGE; the native molecular mass was judged to be 100 kDa by gel filtration on a Superose-12 FPLC column.

Tryptic Peptides of the Exopoly(*P*)ase. The second poly-(*P*)ase was identified as GPP when the partial amino acid sequence in five tryptic peptides matched the GenBank amino acid sequence of GPP (Fig. 1).

Table 1. Purification of an exopoly(*P*)ase from an *E. coli ppx* mutant

			Poly(*P*)ase		pppGpp hydrolase		
Fraction	Step	Total protein, mg	Total activity, units × 10^{-6}	Specific activity, units/mg	Total activity, units × 10^{-6}	Specific activity, units/mg	Poly(*P*)ase/ pppGpp hydrolase ratio
I	Cell lysate	47,000	4.0	85			
II	Ammonium sulfate	34,000	6.8	200			
III	DE52	4,900	4.4	900	0.11	22	40
IV	Phosphocellulose	130	1.2	9,900	0.028	230	43
V	FFQ Sepharose	6	0.23	39,000	0.0049	820	47
VI	Chromatofocusing	0.2	0.053	266,000	0.0014	7000	39

E. coli CA10 was grown to an OD_{595} of 10 in a 200-liter fermenter containing, in g/liter: KH_2PO_4, 4; K_2HPO_4, 16.6; casein hydrolysate, 11; and yeast extract, 22.5. The culture was harvested in a Sharples centrifugal extractor, resuspended in 50 mM Tris·HCl, pH 7.5/10% sucrose/0.1 mM DTT to an OD_{595} of 500, frozen in liquid nitrogen, and stored at −80°C. The cells were moved to −20°C for 24 h prior to lysis. To 900 g of cells was added 450 ml of buffer containing 50 mM Tris·HCl (pH 7.5), 10% sucrose, 200 mM NaCl, 2 mM DTT, 40 mM EDTA, 40 mM spermidine, and lysozyme at 400 μg/ml. The cells were incubated on ice for 3 h, incubated at 37°C for 12 min, incubated on ice for 30 min, sonicated (Branson Sonifier) in 250-ml batches (50% duty cycle, 70% power, 80 pulses total), and centrifuged at 30,100 × *g* for 2 h. The supernatant (fraction I) was decanted, and the pellet was discarded. Ammonium sulfate was added to the supernatant to a concentration of 0.30 g/ml. The mixture was stirred overnight and then centrifuged at 30,100 × *g* for 2 h. The supernatant was decanted and discarded. The pellet was resuspended in 75 ml of 50 mM Tris·HCl, pH 7.5/10 mM KCl/1 mM DTT/10% (vol/vol) glycerol/1 mM $MgCl_2$ and dialyzed two times against 15 liters of buffer A (50 mM Tris·HCl, pH 7.5/10 mM KCl/0.1 mM DTT) for 12 h. A precipitate formed during dialysis and was removed by centrifugation at 30,100 × *g* for 12 h. The supernatant (fraction II) was decanted. DE52 DEAE-cellulose (500 g) was equilibrated with 3 liters of 1 M Tris·HCl (pH 7.5) and then washed several times with buffer A. The protein was loaded at a rate of 2.5 ml/min onto a 1-liter column equilibrated with buffer A, washed with 2 liters of buffer A, and then eluted with a 4-liter gradient from 50 to 500 mM KCl in buffer A. Fractions (50 ml) were collected and assayed. Peak fractions of activity were pooled and dialyzed twice against 10 liters of buffer A. The dialyzed mixture was centrifuged at 30,100 × *g* for 1 h, and the supernatant was collected (fraction III). The dialyzed protein was loaded onto a 750-ml phosphocellulose column equilibrated with buffer B (50 mM Mes–KOH, pH 6.5/10 mM KCl/0.1 mM DTT). The column was washed with 3 liters of buffer B and then eluted with a 4-liter gradient from 10 to 400 mM KCl in this buffer. Fractions (50 ml) were collected, assayed, and pooled. The protein was precipitated with 0.34 g of $(NH_4)_2SO_4$ per ml and centrifuged at 30,100 × *g* for 1 h. The pellet was resuspended in 8 ml of buffer A and dialyzed once against 1 liter of the same buffer for 36 h (fraction IV). The dialyzed protein was loaded onto a 8-ml Fast Flow Q Sepharose column, washed with 40 ml of buffer A, and eluted with an 80-ml gradient from 50 to 500 mM KCl. Fractions (1 ml) were collected, assayed, and pooled (fraction V). The protein was dialyzed against 1 liter of buffer C (25 mM imidazole·HCl, pH 7.4/10 mM KCl/0.1 mM DTT) for 24 h and loaded onto a 5-ml Chromatofocusing column equilibrated to pH 7.4 with buffer C. The column was eluted with 100 ml of 10% Polybuffer 74 (pH 4.0; Pharmacia LKB)/10 mM KCl/0.1 mM DTT. Fractions (1 ml) were collected, assayed, pooled, and equilibrated to pH 7.4 by adding 1 M imidazole·HCl (pH 7.4) to a final concentration of approximately 50 mM (fraction VI).

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MGSTSSLYAA IDLGSNSFHM LVVREVAGSI QTLTRIKRKV RLAAGLNSEN ALSNEAMERG   60
WQCLRLFAER LQDIPPSQIR VVATARLRLA VNAGDFIAKA QEILGCPVQV ISGEEEARLI  120
YQGVAHTTGG ADQRLVVDIG GASTELVTGT GAQTTSLFSL SMGCVTWLER YFADRNLGQE  180
NFDAAEKAAR EVLRPVADEL RYHGWKVCVG ASGTVQALQE IMMAQGMDER ITLEKLQQLK  240
QRAIHCGRLE ELEIDGLTLE RALVFPSGLA ILIAIFTELN IQCMTLAGGA LREGLVYGML  300
HLAVEQDIRS RTLRNIQRRF MIDIDQAQRV AKVAANFFDQ VENEWHLEAI SRDLLISACQ  360
LHEIGLSVDF KQAPQHAAYL VRNLDLPGFT PAQKKLLATL LLNQTNPVDL SSLHQQNAVP  420
PRLAEQLCRL LRLAIIFASR RRDDLVPEMT LQANHELLTL TLPQGWLTQH PLGKEIIAQE  480
SQWQSYVHWP LEVH
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FIG. 1. Correspondence of peptide fragments to the translated *gppA* gene. Tryptic peptides of the purified exopoly(*P*)ase were partially sequenced and identified as the underlined sequences. With the exception of two amino acids in fragment 3 (indicated by the stippled areas), the sequences correspond exactly to those of the *gppA* gene (GenBank accession no. P25552).

Kinetic Properties of the Enzyme. The K_m was determined with [γ-^{32}P]pppGpp as a substrate and was 0.13 mM; for poly(*P*) the K_m was 0.5 nM (as polymer) (Table 2). The k_{cat} for activity (as P_i liberated) on poly(*P*) was 1.1 s^{-1} and on pppGpp it was 0.023 s^{-1}.

Salt Sensitivity of Poly(*P*)ase Activity. The sensitivities to several salts distinguish the poly(*P*)ase activity of GPP from that of PPX (Fig. 2). KCl, NaCl, and NaF inhibited GPP activity, whereas increasing concentrations of the same salts (up to 130 mM) stimulated PPX activity.

GPP Has a Preference for Long-Chain Poly(*P*). The K_m of GPP for long-chain poly(*P*) is approximately 0.5 nM in terms of polymer (assuming a chain length of 500) or 250 nM in terms of P_i residues. Short- to medium-chain poly(*P*)s inhibited GPP feebly in the standard reaction (Fig. 3). P_i and inorganic pyrophosphate (PP_i) had no effect on poly(*P*)ase activity of GPP, whereas P_3, P_4, P_5 reduced activity to 50% when in excess over long-chain [^{32}P]poly(*P*) by 1000- to 10,000-fold. Poly(*P*)s with average chain lengths of 15, 35, and 65 reduced poly(*P*)ase activity to 50% when in excess of [^{32}P]poly(*P*) by 200-fold.

GPP Is Processive. The degree of processivity of GPP on long-chain poly(*P*) was investigated by following the progress of the poly(*P*)ase reaction with time. The products of the reaction were characterized in three ways: chromatography on PEI plates with liquid scintillation counting to measure overall hydrolysis to P_i, electrophoresis on 6% polyacrylamide gels with autoradiography to examine long-chain poly(*P*), and electrophoresis on 20% polyacrylamide gels with autoradiography to examine short- and intermediate-chain poly(*P*)s. Release of P_i from poly(*P*) proceeded to completion over a 60-min period with the disappearance of the long-chain poly(*P*) and the relative absence of progressively shorter chains. Closer examination, by overexposing autoradiograms, revealed the transient appearance of a very low level of a discrete chain size (40 ± 5 residues in length) (Fig. 4*A*). What may be the limit product (4 ± 1 residues in length) appeared late in the reaction. The relative intensities of the bands were quantitated by using densitometry (Fig. 4*B*).

Table 2. K_m and k_{cat} values

Activity	K_m, M × 10^6	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}s^{-1}$ × 10^{-6}
Poly(*P*)ase	0.0005	1.1	2200
pppGpp hydrolase	130	0.023	0.00018

The k_{cat} is defined in terms of the number of phosphate residues liberated per unit time in the standard reaction. The K_m, defined in terms of polymer concentration, was calculated from a Lineweaver–Burk plot.

DISCUSSION

The ubiquity and abundance of poly(*P*) invite attention to the functions of this largely forgotten polymer. Our approach to this problem has been to isolate the enzymes which make and use poly(*P*) in order to (*i*) identify the genes that encode the enzymes, (*ii*) knock out the genes or overexpress them to observe the physiological consequences, (*iii*) determine the distribution of the enzymes in the wild-type and overproducer strains, (*iv*) acquire enzyme reagents of high specificity for the synthesis and analysis of poly(*P*) chains of distinctive lengths and states of complexation, (*v*) explore the enzymatic mechanisms responsible for the metabolism of these remarkable polymers, and (*vi*) apply the biochemical and genetic knowledge to problems as theoretical as prebiotic evolution and as practical as the removal of orthophosphate (P_i) that contaminates waterways.

Our studies of PPK, the enzyme that catalyzes the reversible synthesis of poly(*P*) from ATP, revealed in the *E. coli ppk* operon an adjacent gene (*ppx*) encoding an exopoly(*P*)ase (PPX) that hydrolyzes poly(*P*) to P_i. Mutants that fail to express the *ppx* gene and thus lack PPX were found to contain an exopoly(*P*)ase of lesser activity. This secondary exopoly-

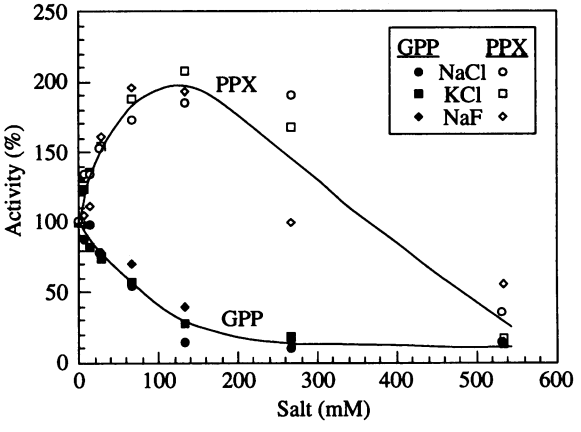

FIG. 2. Effects of salts on GPP and PPX activities The indicated amounts of NaCl, KCl, and NaF were added to the standard poly(*P*)ase assay mixture containing either 50 ng of GPP (fraction VI) or 1 ng of PPX. The enzyme activity is relative to that with no salt addition.

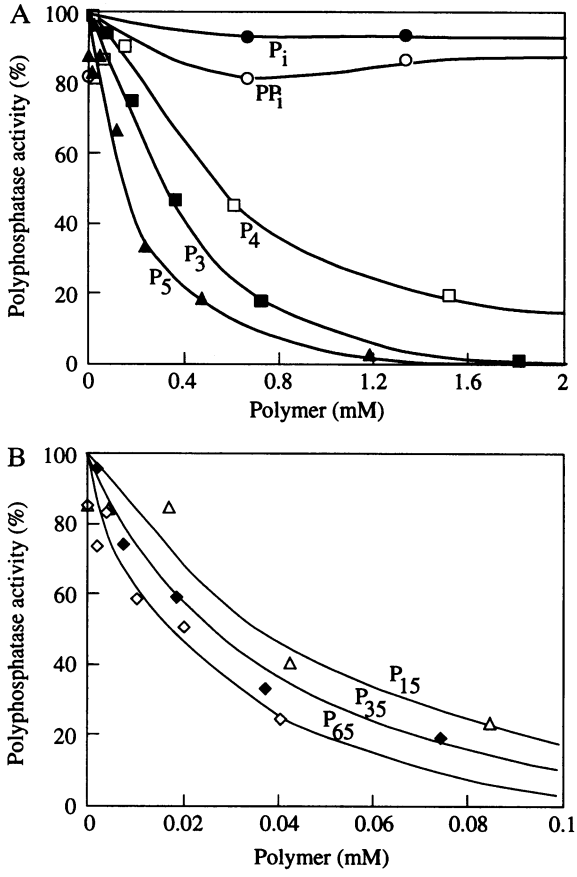

FIG. 3. Effect of short-chain (*A*) and medium-chain (*B*) poly(*P*)s on GPP activity. To the poly(*P*)ase assay were added the indicated amounts of various commercially available poly(*P*)s as the sodium salts. The [^{32}P]poly(*P*) was 0.13 μM in terms of polymer (assuming a chain length of 500). Enzyme activity was measured by the release of [^{32}P]P_i from [^{32}P]poly(*P*) at 30 and 60 min. Poly(*P*)ase activities are relative to those without other additions.

(*P*)ase, when purified to homogeneity, proved astonishingly to be the hydrolase (GPP) that is known to convert pppGpp to ppGpp, the "magic spots" which accumulate in *E. coli* in the "stringent response" to deprivation of an amino acid.

Identity of the secondary exopoly(*P*)ase and the pppGpp phosphohydrolase is based on (*i*) the identity of tryptic digest fragments of the purified enzyme to the translated *gppA* gene (Fig. 1), (*ii*) the same 100-kDa size of the two activities (20), and (*iii*) the constant ratio of the two activities over a 300-fold range of enrichment in the last four steps of purification to homogeneity (Table 1).

While both activities involve the hydrolysis of a phosphoanhydride bond, their kinetic features are quite distinctive. The K_m values are 0.5 nM for poly(*P*) and 0.13 mM for pppGpp, a 2×10^5 difference. The k_{cat} value of 1.1 s^{-1} for poly(*P*) hydrolysis is also near 50-fold greater than the k_{cat} of 0.023 s^{-1} for pppGpp hydrolysis. Thus, the overall potency of the enzyme as an exopoly(*P*)ase would appear to be seven orders of magnitude greater than as a pppGpp hydrolase. However, this discrepancy is more apparent than real in view of two considerations. First, the cellular abundance of pppGpp in the stringent response reaches concentrations (12) which satisfy the high K_m of the hydrolase activity. Second, the higher k_{cat} value for the exopoly(*P*)ase is calculated on the basis of phosphate residues released instead of conversion of the entire poly(*P*) to P_i. This high activity derives from the processivity of the enzyme on the polymeric substrate in which time is not lost by dissociation and reassociation of the substrate.

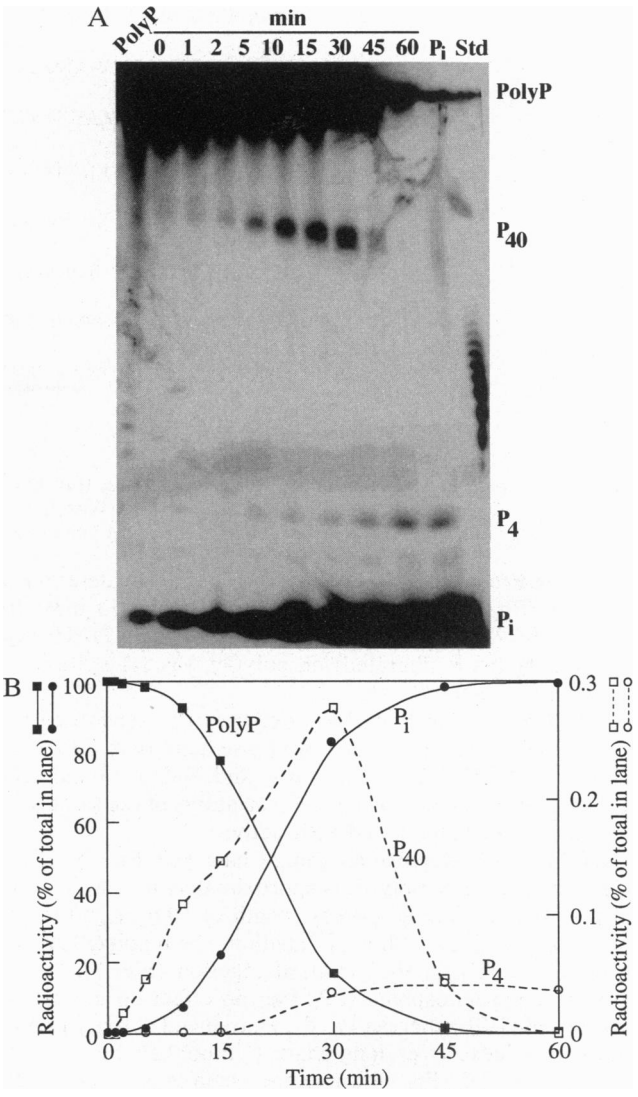

FIG. 4. Progress of the poly(*P*)ase reaction. Activity was measured at 37°C in a 600-μl assay mixture containing 25 mM Hepes–KOH (pH 8.0), 0.5 mM magnesium acetate, 0.5 mM DTT, 66.7 mM ammonium acetate, 1.67 mM [^{32}P]poly(*P*) (in phosphate residues), and 40 μl of fraction VI. Samples (1 μl) were spotted on PEI plates, developed as described in *Materials and Methods*, and quantitated by using liquid scintillation counting. Samples of 45 μl were added to 5 μl of 0.9 M Tris–borate (pH 8.3) and 30 mM EDTA; a portion of each sample (20 μl) was mixed with a marker dye solution (5 μl of 0.25% bromphenol blue and 0.25% xylene cyanol, in 50% glycerol) and loaded onto each of two polyacrylamide gels (pre-electrophoresed at 750 V for 15 min). The gel in *A* contained 20% acrylamide, 2% bisacrylamide, 7 M urea, 89 mM Tris–borate (pH 8.3), 2.7 mM EDTA (pH 8.0), 0.5% ammonium persulfate, and 0.1% *N,N,N′,N′*-tetramethylethylenediamine (TEMED). The gel was run at 750 V until the bromphenol blue had moved 5 cm from the wells. Kodak film was exposed to the gel (not dried; wrapped in Saran Wrap) for 1, 2, 4.5, 7, 12, 15, 68, and 180 h at −80°C. (*A*) Autoradiogram of the 20% polyacrylamide gel exposed to film for 180 h. The right-most lane (Std) is the poly(*P*) ladder. (*B*) Quantitation of the gel at various exposure times using laser densitometry. The radioactivity in each band is plotted as a percentage of the total in each lane. Note the difference in scale between the left and right ordinates.

If the active site for hydrolysis is the same for both activities, then one must account for the enormous difference in the affinities for poly(*P*) and pppGpp as substrates. The processive hydrolysis of the 1000-long chain of poly(*P*) continues until the length is reduced to about 40 residues (17). These intermediate-size chains accumulate until the long

chains are nearly all removed, at which point these too are degraded to P_i and end up as chains of 4 residues. It may be significant that the end products of both poly(*P*) and pppGpp hydrolysis are tetraphosphates.

The recent revelation that the NH_2-terminal domains of PPX and GPP share structural homologies with the ATP-binding clefts of actin and hexokinase (F. Bazan, personal communication) directs attention to the CO_2-terminal regions, which likely serve as recognition domains of these enzymes. Further examination of the sequences of PPX and GPP may provide insights into how GPP distinguishes long-chain from short-chain poly(*P*) and poly(*P*) chains from pppGpp.

The finding that a key enzyme in the stringent response is a long-chain expoly(*P*)ase suggests an involvement of poly(*P*) in cellular adjustments to environmental stresses. Preliminary studies of *E. coli* exposed to serine hydroxamate, a condition that induces the stringent response, confirm earlier qualitative observations of an increase in poly(*P*) (M. Cashel, personal communication).

Still other observations point to the likely importance of poly(*P*) in regulation of gene expression. In the stationary phase in *E. coli*, a marked decrease in transcription and a selection of stress-related promoters over those of biosynthesis genes have been attributed to binding of RNA polymerase by poly(*P*); the holoenzyme isolated from stationary-phase cells displayed the *in vivo* features of the exponential-phase holoenzyme upon conversion of poly(*P*) to ATP by PPK (A. Ishihama, personal communication). Related to these observations may be the poor survival (*sur* phenotype) of *ppk* mutants lacking poly(*P*) (A. Ishihama, personal communication; N. Rao and A.K., unpublished data).

The likely capacity of a strong polyanion like poly(*P*) to bind basic proteins (e.g., histones) and the domains of proteins, such as those of the polymerases that bind polyanionic templates (i.e., RNA and DNA), suggests a variety of regulatory functions in cells. More extensive studies of poly(*P*) in a wide array of cells and tissues should provide clearer views of the ways in which poly(*P*) may be used to effect metabolic changes and determine developmental patterns.

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